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(54) Title: INTERLEUKIN-1 HOMOLOG

(57) Abstract

Homologs of interleukin-1, materials and methods for making them, compositions comprising them, and methods of using them are disclosed. The homologs are proteins comprising a sequence of amino acid residues as shown in SEQ ID NO:7. The proteins have inflammation modulating activity and are useful within related research and therapeutic applications.

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Description

INTERLEUKIN-1 HOMOLOG

BACKGROUND OF THE INVENTION

In multicellular organisms, cell growth, differentiation, migration, and metabolism are controlled by a variety of polypeptide factors. These factors regulate both normal development and pathogenesis.

The interleukins are a family of cytokines that mediate immune and inflammatory responses. Interleukin-1 (IL-1) is a family of at least three known members with recognizable sequence homology and common receptor binding activities. IL-1α and IL-1β are pro-inflammatory cytokines, while the third family member, IL-1 receptor antagonist (IL-1ra) is an antagonist of IL-1α and IL-1β activities. IL-1ra is unusual in that it is the only known, naturally occuring cytokine receptor antagonist with no apparent agonist function. The ability of IL-1ra to bind, but not activate, the IL-1 receptor suggests that IL-1ra is a negative regulator of inflammation (Dripps et al., *J. Biol. Chem.* 266:10331-10336, 1991; Granowitz et al., *Blood* 79:2356-2363, 1992).

The interleukins mediate a variety of inflammatory pathologies. At low concentrations Il-1α and IL-1β act locally on mononuclear phagocytes and the vascular endothelium to induce further IL-1 and IL-6 synthesis. IL-1 does not act directly on leukocytes and neutrophils, but causes the mononuclear phagocytes and endothelial cells to activate leukocytes. When secreted in large quantities into the bloodstream, IL-1 has endocrine effects, including fever, synthesis of acute phase plasma proteins, and cachexia.

Early reports described naturally occurring inhibitors of IL-1 activity in many sources: urine from febrile patients (Seckinger et al., *J. Immunol.* 139:1546-1549, 1987; Mazzei et al., *Eur. J. Immunol.* 20:683-689, 1990), monocytic leukemia patients (Seckinger et al., *ibid.*), or juvenile chronic arthritis patients (Prieur et al., *Lancet* 2:1240-1242, 1987); synovial fluid of rheumatoid arthritis patients (Lotz et al., *J. Clin. Invest.* 78:713-721, 1986); and viral-infected monocytes or B-cells (Scala et al., *J. Exp. Med.* 159:1637-1652, 1984). These inhibitory bioactivities appear to be heterogeneous with a range of molecular mass from 18 to 67 kDa, but they remain largely uncharacterized. The first demonstration of a true IL-1 receptor antagonist

came from the purification and cloning of recombinant IL-1ra from human peripheral blood mononuclear cells (PMNC) cultured on IgG-coated plates and from the human monocytic cell line U937 activated with phorbol ester (PMA) (Carter et al., *Nature* 344:633-638, 1990; Hannum et al., *Nature* 343:336-340, 1990; Eisenberg et al., *Nature* 343:341-346, 1990).

Serum from healthy people has a low level of circulating IL-1ra activity (200-400 pg/ml). Serum IL-1ra levels are dramatically increased in patients with acute or chronic inflammatory disease, certain cancers, infectious diseases, and septic shock (Fisher et al., *Blood* 79:2196-2200, 1992), major surgery for Hirshsprung's disease (O Nuallain et al., *Clin. Exp. Immunol.* 93:218-222, 1993), liver disease (Sekiyama et al., *Clin. Exp. Immunol.* 98:71-77, 1994), Hodgkin's disease (Gruss et al., *Lancet* 340:968, 1992), and colitis (Hyams et al., *Dig. Dis. Sci.* 39:1893-1899, 1994). Other acute and chronic disorders also induce local production of IL-1ra, for example, rheumatoid arthritis (Firestein et al., *J. Immunol.* 149:1054-1062 1992), Lyme arthritis (Miller et al., *Lancet* 341:146-148, 1993), and nonperforating Crohn's disease (Gilberts et al., *Proc. Natl. Acad. Sci. USA* 91: 12721-12724, 1994). Induction of endotoxemia in human volunteers induces 100-fold excesses of IL-1ra over IL-1β in serum, however this level is still apparently insufficient to abolish IL-1 activity *in vivo* (Granowitz et al., *Lancet* 338:1423-1424, 1991). Numerous experiments have shown the potential for systemic, intravenous injection of IL-1ra to attenuate the effects of administered IL-1 or LPS in animal models of endotoxemia or synovitis (Dinarello and Thompson, *Immunol. Today* 12:404-410, 1991). When antibodies to IL-1ra were administered iv in a rabbit formalin-induced colitis model there was significant exacerbation of intestinal inflammation and mortality (Ferretti et al., *J. Clin. Invest.* 94:449-453, 1994).

IL-1ra has been investigated for use in treating several chronic inflammatory disorders including rheumatoid arthritis (Henderson et al., *Cytokine* 3:246-249, 1991), chronic myelogenous leukemia (CML) (Schiro et al., *Blood* 83:460-465, 1994), and inflammatory bowel disease (IBD) (Cominelli et al., *Gastroenterology* 103:65-71, 1992). Some evidence also suggests that IL-1ra may be useful in the treatment of psoriasis. Normal skin expresses IL-1ra mainly in the differentiated stratum granulosum of the epidermis, whereas psoriatic skin expresses IL-1ra in basal midbasal layers (Hammerberg et al, *J. Clin. Invest.* 90:571-583, 1992). Changes in the IL-

1α:IL1-ra ratio in different strata of the epidermis may affect keratinocyte proliferation and differentiation. Chronic inflammatory bowel disease may also involve an altered IL-1:Il-1ra ratio since it is markedly increased in Crohn's disease and ulcerative colitis (Cominelli et al., *Cytokine* 6:A171, 1994). Experimental evidence also suggests that IL-1ra may be useful in chronic and acute cerebral neuropathologies (Relton et al., *Exp. Neurol.* 138:206-213, 1996; Loddick et al., *Biochem. Biophys. Res. Comm.* 234:211-215, 1997), insulin dependent diabetes mellitus (Madrup-Poulsen et al., *Cytokine* 5:185-191, 1993), glomerulonephritis (Lan et al., *Kidney Int.* 47:1303-1309, 1995), and pancreatitis (Norman et al., *Ann. Surg.* 221:625, 1995).

Increased IL-1 production has been reported in patients with various viral, bacterial, fungal, and parasitic infections; intravascular coagulation; high-dose IL-2 therapy; solid tumors; leukemias; Alzheimer's disease; HIV-1 infection; autoimmune disorders; trauma (surgery); hemodialysis; ischemic diseases (myocardial infarction); noninfectious hepatitis; asthma; UV radiation; closed head injury; pancreatitis; peridontitis; graft-versus-host disease; transplant rejection; and in healthy subjects after strenuous exercise (Dinarello, *Blood* 87:2095-2147, 1996).

Recombinant IL-1ra has been shown to be well tolerated in clinical trials in humans (Campion et al., *Arthritis and Rhematism* 39: 1092-1101, 1996), and to be potentially efficacious in the treatment of septic shock (Fisher et al., *JAMA* 271:1836-1843, 1994), rheumatoid arthritis (Campion et al., *Arthritis & Rheumatism* 39:1092-1101, 1996), and graft vs. host disease (GVHD) (Antin et al., *Blood* 84:1342-1348, 1994). However, high serum concentrations of the molecule are often required because of receptor binding affinity, plasma half-life, and tissue permeability. There thus remains a need in the art for molecules that control inflammatory processes.

SUMMARY OF THE INVENTION

The present invention provides novel interleukin-1 (IL-1) homologs, as well as materials and methods for making the IL-1 homologs, compositions comprising them, and methods for using them. Within one of its aspects, the invention thus provides an isolated protein comprising a sequence of amino acid residues as shown in SEQ ID NO:7. Within certain embodiments of the invention, the protein comprises a Lys residue at position 148 of SEQ ID NO:7 or an Asp residue at position 148 of SEQ ID NO:7.

Within another embodiment, the protein comprises the amino acid sequence of SEQ ID NO:8. Within other embodiments, the protein is from 155 to 1200 amino acid residues in length. Within further embodiments, the protein is 155 amino acid residues in length.

Within a second aspect of the invention there is provided an isolated polypeptide of at least 15 amino acid residues comprising an epitope-bearing portion of a protein of SEQ ID NO:7.

Within a third aspect of the invention there is provided an expression vector comprising the following operably linked elements: (a) a transcription promoter; (b) a DNA segment encoding a protein comprising a sequence of amino acid residues as shown in SEQ ID NO:7; and (c) a transcription terminator. Within one embodiment, the expression vector further comprises a secretory signal sequence operably linked to the DNA segment. Within other embodiments, the protein comprises a Lys residue or an Asp residue at position 148 of SEQ ID NO:7. Within another embodiment, the protein comprises the amino acid sequence of SEQ ID NO:8. Within other embodiments, the protein is from 155 to 1200 amino acid residues in length. Within further embodiments, the protein is 155 amino acid residues in length.

Within a fourth aspect of the invention there is provided a cultured cell comprising an expression vector as disclosed above.

Within a fifth aspect, the invention provides a method of making a protein, comprising culturing a cell as disclosed above under conditions wherein the DNA segment is expressed, and recovering the protein encoded by the DNA segment.

Within a sixth aspect, the invention provides an antibody that specifically binds to a protein as disclosed above.

Within a seventh aspect of the invention there is provided a method of modulating an immune response in an animal comprising administering to the animal a composition comprising a protein as disclosed above in combination with a pharmaceutically acceptable vehicle.

These and other aspects of the invention will become evident upon reference to the following detailed description of the invention and the attached drawing.

BRIEF DESCRIPTION OF THE DRAWING

The figure is a Hopp/Woods hydrophilicity profile of the amino acid sequence shown in SEQ ID NO:2. The profile is based on a sliding six-residue window. Buried G, S, and T residues and exposed H, Y, and W residues were ignored. These residues are indicated in the figure by lower case letters.

DETAILED DESCRIPTION OF THE INVENTION

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson et al., *EMBO J.* 4:1075, 1985; Nilsson et al., *Methods Enzymol.* 198:3, 1991), glutathione S transferase (Smith and Johnson, *Gene* 67:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., *Proc. Natl. Acad. Sci. USA* 82:7952-4, 1985) (SEQ ID NO:13), substance P, Flag™ peptide (Hopp et al., *Biotechnology* 6:1204-1210, 1988), streptavidin binding peptide, maltose binding protein (Guan et al., *Gene* 67:21-30, 1987), cellulose binding protein, thioredoxin, ubiquitin, T7 polymerase, or other antigenic epitope or binding domain. See, in general, Ford et al., *Protein Expression and Purification* 2: 95-107, 1991. DNAs encoding affinity tags and other reagents are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ; New England Biolabs, Beverly, MA; Eastman Kodak, New Haven, CT).

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain

sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

A "complement of a polynucleotide molecule" is a polynucleotide molecule having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "corresponding to", when applied to positions of amino acid residues in sequences, means corresponding positions in a plurality of sequences when the sequences are optimally aligned.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "expression vector" is used to denote a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, *Nature* 316:774-78, 1985).

An "isolated" polypeptide or protein is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

A "motif" is a series of amino acid positions in a protein sequence for which certain amino acid residues are required. A motif defines the set of possible residues at each such position.

The term "operably linked", when referring to DNA segments, indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

"Paralogs" are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example, α-globin, β-globin, and myoglobin are paralogs of each other.

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded

polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Many cell-surface receptors are, in their active forms, multi-peptide structures in which the ligand-binding and signal transduction functions may reside in separate subunits. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids, and hydrolysis of phospholipids. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF

receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

A "segment" is a portion of a larger molecule (e.g., polynucleotide or polypeptide) having specified attributes. For example, a DNA segment encoding a specified polypeptide is a portion of a longer DNA molecule, such as a plasmid or plasmid fragment, that, when read from the 5' to the 3' direction, encodes the sequence of amino acids of the specified polypeptide.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a protein encoded by a splice variant of an mRNA transcribed from a gene.

Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to ±10%.

The present invention provides a group of novel proteins, designated "zil1a3", that are members of the IL-1 family. Analysis of the human zil1a3 sequence (SEQ ID NO:1 and NO:2) indicates that this protein, like other members of the family, contains a core structure of 12 β-strands wound into a β-barrel, with the β-strands separated from each other by loops. The loops between these β-strands are highly variable among the family members and are believed to be involved in receptor binding. These loops, which each contain at least three amino acid residues and may contain up to 17 residues, do not form β-strands or helices, but may (and often do) contain

β-turns. Referring to SEQ ID NO:2, the twelve β-strands are formed by residues 8-13, 17-21, 26-28, 41-47, 56-61, 66-71, 77-83, 99-105, 109-113, 119-123, 131-133, and 149-153. These strands are characterized by a high content of hydrophobic amino acid residues (Leu, Val, Phe, and Ile). The loops include residues 14-16, 22-25, 29-40, 48-55, 62-65, 72-76, 84-98, 106-108, 114-118, 124-130, and 134-148.

In addition to the β-strands and loops, the zil1a3 proteins are characterized by the presence of conserved motifs at positions corresponding to (1) residues 111-115 of SEQ ID NO:2, (2) residues 117-121 of SEQ ID NO:2, (3) residues 130-134 of SEQ ID NO:2, and (4) residues 146-149 of SEQ ID NO:2. These motifs are shown in Table 1 using the standard single-letter codes for amino acid residues.

Table 1

Motif	Sequence	SEQ ID NO:
1	F E/T S A/V A/Q	3
2	P G/N L/W F/Y I/L	4
3	P/W V/L S/C/F/R/I L T/A/G	5
4	V/I T K/D/E F	6

The higher-order structure of the IL-1 family of proteins can also be envisioned as three 4-stranded covalent monomers assembled into a 12-stranded structure (a "trefoil"). When these monomers are superimposed on each other, residues 41, 99, and 148 of SEQ ID NO:2, SEQ ID NO:7, and SEQ ID NO:8 occupy like positions in their respective monomers.

The proteins of the present invention have pro-inflammatory (agonist) or anti-inflammatory (antagonist) activity, depending on the particular amino acid sequence. In general, zil1a3 proteins having a Lys residue corresponding to position 148 of SEQ ID NO:2 will have anti-inflammatory activity, while those having an Asp or Glu residue at this position will have pro-inflammatory activity. Sequence variations at positions corresponding to residues 41 and 99 of SEQ ID NO:2 may also influence biological activity. Within certain embodiments the present invention includes zil1a3 proteins having Lys or Glu at a position corresponding to residue 41 of SEQ ID NO:2

and Ala, Ile, or Thr at a position corresponding to residue 99 of SEQ ID NO:2. These sequence variations within human zil1a3 are represented in SEQ ID NO:7. While not wishing to be bound by theory, it is believed that zil1a3 proteins act through IL-1 receptors. Pro- and anti-inflammatory activities can be assayed using standard assays of IL-1 activity known in the art.

A limited number of non-conservative amino acid substitutions, amino acids that are not encoded by the genetic code, and non-naturally occurring amino acids may be incorporated into a zil1a3 protein. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., *J. Am. Chem. Soc.* 113:2722, 1991; Ellman et al., *Methods Enzymol.* 202:301, 1991; Chung et al., *Science* 259:806-9, 1993; and Chung et al., *Proc. Natl. Acad. Sci. USA* 90:10145-9, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., *J. Biol. Chem.* 271:19991-8, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide et al., *Biochem.* 33:7470-6, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro*

chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci.* 2:395-403, 1993).

Those skilled in the art will recognize that additional amino acid sequence changes can be made in the zil1a3 sequence shown in SEQ ID NO:7 to obtain other zil1a3 proteins. These changes are made so as to minimize disruption of higher order structure essential to biological activity. In particular, the arrangement of β-strands and loops will not be disrupted, thus it is preferred to make conservative amino acid substitutions within the β-strands, particularly when replacing hydrophobic residues. See Table 2. Those skilled in the art will recognize that the categories within Table 2 are somewhat arbitrary, and that hydrophobic and aromatic residues can sometimes substitute for each other in a sequence. For example, phenylalanine occurs in motifs 2, 3, and 4 of zil1a3, where it is believed to serve a hydrophobic function. The effects of amino acid sequence changes can be predicted by computer modeling using available software (e.g., the Insight II® viewer and homology modeling tools; MSI, San Diego, CA) or determined by alignment and analysis of crystal structures. See, Priestle et al., *EMBO J.* 7:339-343, 1988; Priestle et al., *Proc. Natl. Acad. Sci. USA* 86:9667-9671, 1989; Finzel et al., *J. Mol. Biol.* 209:779-791, 1989; Graves et al., *Biochem.* 29:2679-2684, 1990; Clore and Gronenborn, *J. Mol. Biol.* 221:47-53, 1991; Vigers et al., *J. Biol. Chem.* 269:12874-12879, 1994; Schreuder et al., *Eur. J. Biochem.* 227:838-847, 1995; and Schreuder et al., *Nature* 386:194-200, 1997. A hydrophilicity profile of SEQ ID NO:2 is shown in the attached figure. Those skilled in the art will recognize that hydrophobicity and hydrophilicity will be taken into account when designing alterations in the amino acid sequence of a zil1a3 polypeptide, so as not to disrupt the overall profile. Alignment of zil1a3 with other family members also provides guidance in selecting amino acid substitutions, particularly if information about the effects of amino acid substitutions in other family members is available. For example, alignment suggests that residue 148 (Asp) can be replaced with Lys, resulting in a change in activity from agonist to antagonist (Ju et al., *Proc. Natl. Acad. Sci. USA* 88:2658-2662, 1991; Oldfield et al., *Protein Eng.* 6:865-871, 1993). This variant of SEQ ID NO:2 (designated "hzil1a3-D148K") is shown in SEQ ID NO:8. It is in general preferred not to alter the sequences of the loops because these regions of the

molecule are believed to be important for receptor binding, although residues 114, 115, 132, 134, 146, and 148 of SEQ ID NO:2 may be substituted within the boundaries of the motifs defined herein. However, alteration of the loops can be used to generate tools for investigation of receptor binding specificity and other aspects of interleukin biology.

Table 2

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

The proteins of the present invention can further comprise amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or an affinity tag as disclosed above. Two or more affinity tags may be used in combination. Polypeptides comprising affinity tags can further comprise a polypeptide linker and/or a proteolytic cleavage site between the zil1a3 polypeptide and the affinity tag. Preferred cleavage sites include thrombin cleavage sites and factor Xa cleavage sites.

The present invention further provides a variety of other polypeptide fusions. For example, a zil1a3 polypeptide can be prepared as a fusion to a dimerizing protein as disclosed in U.S. Patents Nos. 5,155,027 and 5,567,584. Preferred dimerizing proteins in this regard include immunoglobulin constant region domains. Immunoglobulin-zil1a3 polypeptide fusions can be expressed in genetically engineered cells to produce a variety of multimeric zil1a3 analogs. In addition, a zil1a3 polypeptide can be joined to another bioactive molecule, such as a cytokine, to provide a multi-functional molecule. One or more helices of a zil1a3 polypeptide can be joined to another cytokine to enhance or otherwise modify its biological properties. Auxiliary domains can be fused to zil1a3 polypeptides to target them to specific cells, tissues, or macromolecules (e.g., collagen). For example, a zil1a3 polypeptide or protein can be targeted to a predetermined cell type by fusing a zil1a3 polypeptide to a ligand that specifically binds to a receptor on the surface of the target cell. In this way, polypeptides and proteins can be targeted for therapeutic or diagnostic purposes. A zil1a3 polypeptide can be fused to two or more moieties, such as an affinity tag for purification and a targeting domain. Polypeptide fusions can also comprise one or more cleavage sites, particularly between domains. See, Tuan et al., *Connective Tissue Research* 34:1-9, 1996.

Polypeptide fusions of the present invention will generally contain not more than about 1,500 amino acid residues, preferably not more than about 1,200 residues, more preferably not more than about 1,000 residues, and will in many cases be considerably smaller. For example, a zil1a3 polypeptide of 155 residues (residues 1-155 of SEQ ID NO:2) can be fused to *E. coli* β-galactosidase (1,021 residues; see Casadaban et al., *J. Bacteriol.* 143:971-980, 1980), a 10-residue spacer, and a 4-residue factor Xa cleavage site to yield a polypeptide of 1,190 residues. In a second example, residues 1-155 of SEQ ID NO:2 can be fused to maltose binding protein (approximately 370 residues), a 4-residue cleavage site, and a 6-residue polyhistidine tag.

Essential amino acids in the proteins of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244, 1081-1085, 1989; Bass et al., *Proc. Natl. Acad. Sci. USA* 88:4498-4502, 1991). In the latter technique, single alanine mutations are

introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity or other properties to identify amino acid residues that are critical to the activity of the molecule.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (*Science* 241:53-57, 1988) or Bowie and Sauer (*Proc. Natl. Acad. Sci. USA* 86:2152-2156, 1989). These authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., *Biochem.* 30:10832-10837, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., *Gene* 46:145, 1986; Ner et al., *DNA* 7:127, 1988).

Variants of the disclosed zil1a3 DNA and polypeptide sequences can be generated through DNA shuffling as disclosed by Stemmer, *Nature* 370:389-391, 1994 and Stemmer, *Proc. Natl. Acad. Sci. USA* 91:10747-10751, 1994. Briefly, variant genes are generated by *in vitro* homologous recombination by random fragmentation of a parent gene followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent genes, such as allelic variants or genes from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed above can be combined with high volume or high-throughput screening methods to detect biological activity of zil1a3 variant proteins. Mutagenized DNA molecules that encode active zil1a3 polypeptides can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure. Assays for IL-1 biological activity and receptor binding are known in the art. Exemplary activity assays include mitogenesis assays in which IL-1

responsive cells (e.g., D10.N4.M cells) are incubated in the presence of IL-1 or a test zil1a3 protein for 72 hours at 37°C in a 5% CO_2 atmosphere. IL-2 (and optionally IL-4) is added to the culture medium to enhance sensitivity and specificity of the assay. [^{3}H]thymidine is then added, and incubation is continued for six hours. The amount of label incorporated is indicative of agonist activity. See, Hopkins and Humphreys, *J. Immunol. Methods* 120:271-276, 1989; Greenfeder et al., *J. Biol. Chem.* 270:22460-22466, 1995. IL-1 stimulation of cell proliferation can also be measured using thymocytes cultured in IL-1 in combination with phytohemagglutinin. Proliferation is detected as [^{3}H]thymidine incorporation or through the use of a colorimetric assay based on the metabolic breakdown of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mosman, *J. Immunol. Meth.* 65: 55-63, 1983). Briefly, a solution of MTT is added to 100 μl of assay cells, and the cells are incubated at 37° C. After 4 hours, 200 μl of 0.04 N HCl in isopropanol is added, the solution is mixed, and the absorbance of the sample is measured at 570 nm. Receptor binding can be measured by the competition binding method of Labriola-Tompkins et al., *Proc. Natl. Acad. Sci. USA* 88:11182-11186, 1991. Briefly, membranes pepared from EL-4 thymoma cells (Paganelli et al., *J. Immunol.* 138:2249-2253, 1987) are incubated in the presence of the test protein for 30 minutes at 37°C. Labeled IL-1α or IL-1β is then added, and the incubation is continued for 60 minutes. The assay is terminated by membrane filtration. The amount of bound label is determined by conventional means (e.g., γ counter). In an alternative assay, the ability of a zil1a3 protein to compete with labeled IL-1 for binding to cultured human dermal fibroblasts is measured according to the method of Dower et al. (*Nature* 324:266-268, 1986). Briefly, cells are incubated in a round-bottomed, 96-well plate in a suitable culture medium (e.g., RPMI 1640 containing 1% BSA, 0.1% Na azide, and 20 mM HEPES pH 7.4) at 8°C on a rocker platform in the presence of labeled IL-1. Various concentrations of zil1a3 protein are added. After the incubation (typically about two hours), cells are separated from unbound label by centrifuging 60-μl aliquots through 200 μl of phthalate oils in 400-μl polyethylene centrifuge tubes and excising the tips of the tubes with a razor blade as disclosed by Segal and Hurwitz, *J. Immunol.* 118:1338-1347, 1977. Receptor binding can also be measured using immobilized receptors or ligand-binding receptor fragments. For example, an immobilized IL-1 receptor can be exposed to labeled IL-1 and

unlabeled test protein, whereby a reduction in IL-1 binding compared to a control is indicative of receptor-binding activity in the test protein. Within another format, a receptor or ligand-binding receptor fragment is immobilized on a biosensor (e.g., BIACore™, Pharmacia Biosensor, Piscataway, NJ) and binding is determined. Cloned cDNAs encoding mouse and human IL-1 receptors are disclosed by Dower et al., U.S. Patent No. 5,081,228. IL-1 antagonists will exhibit receptor binding but will exhibit essentially no activity in IL-1 activity assays or will reduce the IL-1-mediated response when combined with IL-1. In view of the low level of receptor occupancy required to produce a response to IL-1, a large excess of antagonist (typically a 10- to 1000-fold molar excess) may be necessary to neutralize IL-1 activity.

Using the methods disclosed above, one of ordinary skill in the art can identify and/or prepare a variety of zil1a3 proteins having IL-1 agonist or antagonist activity. Such polypeptides can also include additional polypeptide segments as generally disclosed above.

The present invention further provides polynucleotide molecules, including DNA and RNA molecules, encoding zil1a3 proteins. The polynucleotides of the present invention include the sense strand; the anti-sense strand; and the DNA as double-stranded, having both the sense and anti-sense strand annealed together by hydrogen bonds. A representative DNA sequence encoding a human zil1a3 protein is set forth in SEQ ID NO:1. DNA sequences encoding other zil1a3 proteins can be readily generated by those of ordinary skill in the art based on the genetic code. Counterpart RNA sequences can be generated by substitution of U for T.

Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:9 is a degenerate DNA sequence that encompasses all DNAs that encode the zil1a3 polypeptide of SEQ ID NO:2. SEQ ID NO:10 is a degenerate DNA sequence that encompasses all DNAs that encode the zil1a3 polypeptide of SEQ ID NO:8. Those skilled in the art will recognize that the degenerate sequences of SEQ ID NO:9 and SEQ ID NO:10 also provide all RNA sequences encoding SEQ ID NO:2 and SEQ ID NO:8, respectively, by substituting U for T. Thus, zil1a3 polypeptide-encoding polynucleotides comprising nucleotide 1 to nucleotide 465 of SEQ ID NO:9, nucleotide 1 to nucleotide 465 of SEQ ID NO:10, and their respective RNA equivalents are contemplated by the present invention.

Table 3 sets forth the one-letter codes used within SEQ ID NOS:9 and 10 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C or T, and its complement R denotes A or G, A being complementary to T, and G being complementary to C.

TABLE 3

Nucleotide	Resolution	Nucleotide	Complement
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A|G	Y	C|T
Y	C|T	R	A|G
M	A|C	K	G|T
K	G|T	M	A|C
S	C|G	S	C|G
W	A|T	W	A|T
H	A|C|T	D	A|G|T
B	C|G|T	V	A|C|G
V	A|C|G	B	C|G|T
D	A|G|T	H	A|C|T
N	A|C|G|T	N	A|C|G|T

The degenerate codons used in SEQ ID NOS:9 and 10, encompassing all possible codons for a given amino acid, are set forth in Table 4.

TABLE 4

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn|Asp	B		RAY
Glu|Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by a degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence shown in SEQ ID NO:2. Variant sequences can be readily tested for functionality as described herein.

One of ordinary skill in the art will also appreciate that different species can exhibit preferential codon usage. See, in general, Grantham et al., *Nuc. Acids Res.* 8:1893-912, 1980; Haas et al. *Curr. Biol.* 6:315-24, 1996; Wain-Hobson et al., *Gene* 13:355-64, 1981; Grosjean and Fiers, *Gene* 18:199-209, 1982; Holm, *Nuc. Acids Res.* 14:3075-87, 1986; and Ikemura, *J. Mol. Biol.* 158:573-97, 1982. Preferred codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferred codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Therefore, the degenerate codon sequences disclosed in SEQ ID NOS:9 and 10 serve as templates for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein. Sequences containing preferred codons can be tested and optimized for expression in various host cell species, and tested for functionality as disclosed herein.

As previously noted, zil1a3 polynucleotides provided by the present invention include DNA and RNA. Methods for preparing DNA and RNA are well known in the art. In general, RNA is isolated from a tissue or cell that produces large amounts of zil1a3 RNA. Such tissues and cells are identified by Northern blotting (Thomas, Proc. Natl. Acad. Sci. USA 77:5201, 1980), and include stomach and skin. Total RNA can be prepared using guanidine-HCl extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et al., *Biochemistry* 18:52-94, 1979). Poly $(A)^+$ RNA is prepared from total RNA using the method of Aviv and Leder (*Proc. Natl.*

Acad. Sci. USA 69:1408-12, 1972). Complementary DNA (cDNA) is prepared from poly(A)$^+$ RNA using known methods. In the alternative, genomic DNA can be isolated. Polynucleotides encoding zil1a3 polypeptides are then identified and isolated by, for example, hybridization or PCR.

The polynucleotides of the present invention can also be synthesized using automated equipment ("gene machines") according to methods known in the art. See, for example, Glick and Pasternak, Molecular Biotechnology, Principles & Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994; Itakura et al., *Annu. Rev. Biochem.* 53: 323-356, 1984; and Climie et al., *Proc. Natl. Acad. Sci. USA* 87:633-637, 1990.

The zil1a3 polynucleotide sequences disclosed herein can be used to isolate polynucleotides encoding other zil1a3 proteins. Such other polynucleotides include alternatively spliced cDNAs (including cDNAs encoding secreted zil1a3 proteins) and counterpart polynucleotides from other species (orthologs). These orthologous polynucleotides can be used, *inter alia*, to prepare the respective orthologous proteins. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses zil1a3 as disclosed herein. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A zil1a3-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. Hybridization will generally be done under low stringency conditions, wherein washing is carried out in 1 x SSC with an initial wash at 40°C and with subsequent washes at 5°C higher intervals until background is suitably reduced. A cDNA can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent No. 4,683,202), using primers designed from the representative human zil1a3 sequence disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to zil1a3 polypeptide. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that the sequences disclosed in SEQ ID NO:1 and SEQ ID NO:2 represent a single allele of human zil1a3, and that natural variation, including allelic variation and alternative splicing, is expected to occur. Allelic variants of these sequences

can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the DNA sequence shown in SEQ ID NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO:2. cDNAs generated from alternatively spliced mRNAs that retain the inflammation modulating activity of zil1a3 are included within the scope of the present invention, as are polypeptides encoded by such cDNAs and mRNAs. For example, the sequence disclosed in SEQ ID NO:1 and SEQ ID NO:2 is believed to represent a cytoplasmically expressed form of the protein in view of the lack of a signal peptide sequence. There is expected to be an alternately spliced form of the DNA which includes a secretory signal sequence. An additional, larger band is seen on northern blots, indicating the existence of such an alternate form. Within the human DNA sequence (SEQ ID NO:2), sequence upstream of the initiation ATG may encode an alternatively spliced form of the protein or an alternative form that is translated from an upstream ATG.

Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

The proteins of the present invention, including full-length proteins and fusion proteins, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al., eds., *Current Protocols in Molecular Biology*, Green and Wiley and Sons, NY, 1993.

In general, a DNA sequence encoding a zil1a3 protein is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more

selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a zil1a3 protein into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of a zil1a3 gene, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is operably linked to the zil1a3 DNA sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly sythesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830). In the alternative, a zil1a3 protein is expressed cytoplasmically and is isolated after lysing the host cells.

Cultured mammalian cells are suitable hosts for use within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., *Cell* 14:725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7:603, 1981: Graham and Van der Eb, *Virology* 52:456, 1973), electroporation (Neumann et al., *EMBO J.* 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., *ibid.*), and liposome-mediated transfection (Hawley-Nelson et al., *Focus* 15:73, 1993; Ciccarone et al., *Focus* 15:80, 1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed by, for example, Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573;

Graham et al., *J. Gen. Virol.* 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." An exemplary selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. An exemplary amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g. hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternative markers that produce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, and placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., *J. Biosci. (Bangalore)* 11:47-58, 1987. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222 and WIPO publication WO 94/06463.

Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV). See, King and Possee, The Baculovirus Expression System: A Laboratory Guide, London, Chapman & Hall; O'Reilly et al., Baculovirus Expression Vectors: A Laboratory Manual, New York, Oxford University Press., 1994; and Richardson, Ed., Baculovirus Expression Protocols. Methods in Molecular Biology, Humana Press, Totowa, NJ, 1995. Recombinant baculovirus can also be produced through the use of a transposon-based system described by Luckow et al. (*J. Virol.* 67:4566-4579, 1993). This system, which utilizes transfer vectors, is commercially available in kit form (Bac-to-Bac™ kit; Life Technologies, Rockville, MD). The transfer vector (e.g., pFastBac1™; Life Technologies) contains a Tn7 transposon to move the DNA encoding the protein of interest into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." See, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971-976, 1990; Bonning et al., *J. Gen. Virol.* 75:1551-1556, 1994; and Chazenbalk and Rapoport, *J. Biol. Chem.* 270:1543-1549, 1995. In addition, transfer vectors can include an in-frame fusion with DNA encoding a polypeptide extension or affinity tag as disclosed above. Using techniques known in the art, a transfer vector containing a zil1a3-encoding sequence is transformed into *E. coli* host cells, and the cells are screened for bacmids which contain an interrupted lacZ gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, such as Sf9 cells. Recombinant virus that expresses zil1a3 protein is subsequently produced. Recombinant viral stocks are made by methods commonly used the art.

For protein production, the recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda* (e.g., Sf9 or Sf21 cells) or *Trichoplusia ni* (e.g., High Five™ cells; Invitrogen, Carlsbad, CA). See, in general, Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994. See also, U.S. Patent No. 5,300,435. Serum-free media are used to grow and maintain the cells. Suitable media formulations are known in the art and can be obtained from commercial suppliers. The cells are grown up from an inoculation density of approximately 2-5 x 10^5 cells to a density of 1-2 x 10^6 cells, at which time a recombinant viral stock is added

at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. Procedures used are generally described in available laboratory manuals (e.g., King and Possee, *ibid.*; O'Reilly et al., *ibid.*; Richardson, *ibid.*).

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces fragilis, Ustilago maydis, Pichia pastoris, Pichia methanolica, Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., *J. Gen. Microbiol.* 132:3459-3465, 1986; Cregg, U.S. Patent No. 4,882,279; and Raymond et al., *Yeast* 14, 11-23, 1998. Aspergillus cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228. Methods for transforming Neurospora are disclosed by Lambowitz, U.S. Patent No. 4,486,533. Production of recombinant proteins in *Pichia methanolica* is disclosed in U.S. Patents No. 5,716,808, 5,736,383, 5,854,039, and 5,888,768; and WIPO publications WO 99/14347 and WO 99/14320.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the

present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al., *ibid.*). When expressing a zil1a3 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the protein is recovered from the soluble fraction. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

It is preferred to purify the proteins of the present invention to ≥80% purity, more preferably to ≥90% purity, even more preferably ≥95% purity, and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, a purified protein is substantially free of other polypeptides or proteins, particularly those of animal origin.

Expressed recombinant zil1a3 proteins (including fusion proteins) are purified by conventional protein purification methods, typically by a combination of chromatographic techniques. See, in general, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988; and Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York, 1994. Proteins comprising a polyhistidine affinity tag (typically about 6 histidine residues) are purified by affinity chromatography on a nickel chelate resin. See, for example, Houchuli

et al., *Bio/Technol.* 6: 1321-1325, 1988. Proteins comprising a glu-glu tag can be purified by immunoaffinity chromatography according to conventional procedures. See, for example, Grussenmeyer et al., *ibid.* Maltose binding protein fusions are purified on an amylose column according to methods known in the art.

Zil1a3 polypeptides can also be prepared through chemical synthesis according to methods known in the art, including exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. See, for example, Merrifield, *J. Am. Chem. Soc.* 85:2149, 1963; Stewart et al., Solid Phase Peptide Synthesis (2nd edition), Pierce Chemical Co., Rockford, IL, 1984; Bayer and Rapp, *Chem. Pept. Prot.* 3:3, 1986; and Atherton et al., Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford, 1989. *In vitro* synthesis is particularly advantageous for the preparation of smaller polypeptides.

Zil1a3 proteins of the present invention can be used to modulate inflammation and related processes. Of particular interest is the reduction of inflammation by antagonist forms of zil1a3 (e.g., SEQ ID NO:8). Thus, certain zil1a3 proteins may be used to treat or prevent chronic inflammatory diseases such as arthritis (including rheumatoid arthritis, osteoarthritis, and Lyme arthritis) and psoriasis; to reduce tissue damage after ischemia; and to treat septic shock, graft-versus-host disease, and leukemia. As used herein, the terms "treat" and "treatment" will be understood to include the reduction of symptoms as well as effects on the underlying disease processes. Antagonists are expected to have *in vivo* activity like of that of IL-1 receptor antagonist (IL-1ra), which has shown beneficial effects in clinical trials directed to the treatment of rheumatoid arthritis (Campion et al., *Arthritis & Rheumatism* 39:1092-1101, 1996), graft-versus-host disease (Antin et al., *Blood* 84:1342-1348, 1994), septic shock (Fisher et al., *JAMA* 271:1836-1843, 1994), and leukemia (Dinarello, *Blood* 87:2095-2147, 1996). Experimental data also suggest that antagonism of IL-1 activity will prove beneficial in the treatment of inflammatory bowel disease (including Crohn's disease and ulcerative colitis) (reviewed by Hendel et al., *Exp. Opin. Invest. Drugs* 5:843-850, 1996; see also, Cominelli et al., *Gastroenterology* 103:65-71, 1992), insulin-dependent diabetes mellitus (reviewed by Mandrup-Poulsen et al., *Cytokine* 5:185-191, 1993; see also, Dayer-Metroz et al., *Eur. J. Clin. Inv.* 22:A50, 1992), acute pancreatitis (Norman et al., *Ann. Surg.* 221:625-634,

1995), glomerulonephritis (Lan et al., *Kidney Int.* 47:1303-1309, 1995), and cerebral ischemia (Relton et al., *Exp. Neurology* 138:206-213, 1996; Loddick et al., *Biochem. Biophys. Res. Comm.* 234:211-215, 1997). Agonists may promote wound healing in view of the effects of IL-1 on growth factor secretion and cell proliferation or be useful in the treatment of infections, in particular gastrointestinal infections.

Zil1a3 proteins can be tested in animal models of disease. Animal models of psoriasis include the analysis of histological alterations in adult mouse tail epidermis (Hofbauer et al, *Brit. J. Dermatol.* 118:85-89, 1988; Bladon et al., *Arch Dermatol. Res.* 277:121-125, 1985). In this model, anti-psoriatic activity is indicated by the induction of a granular layer and orthokeratosis in areas of scale between the hinges of the tail epidermis. Typically, a topical ointment is applied daily for seven consecutive days, then the animal is sacrificed, and tail skin is examined histologically. An additional model is provided by grafting psoriatic human skin to congenitally athymic (nude) mice (Krueger et al., *J. Invest. Dermatol.* 64:307-312, 1975). Such grafts have been shown to retain the characteristic histology for up to eleven weeks. As in the mouse tail model, the test composition is applied to the skin at predetermined intervals for a period of one to several weeks, at which time the animals are sacrificed and the skin grafts examined histologically. A third model has been disclosed by Fretland et al. (*Inflammation* 14:727-739, 1990; incorporated herein by reference). Briefly, inflammation is induced in guinea pig epidermis by topically applying phorbol ester (phorbol-12-myristate-13-acetate; PMA), typically at ca. 2 g/ml in acetone, to one ear and vehicle to the contralateral ear. Test compounds are applied concurrently with the PMA, or may be given orally. Histological analysis is performed at 96 hours after application of PMA. This model duplicates many symptoms of human psoriasis, including edema, inflammatory cell diapedesis and infiltration, high LTB_4 levels and epidermal proliferation. Cerebral ischemia can be studied in a rat model as disclosed by Relton et al. (*ibid.*) and Loddick et al. (*ibid.*). Wound-healing models include the linear skin incision model of Mustoe et al. (*Science* 237:1333, 1987). In a typical procedure, a 6-cm incision is made in the dorsal pelt of an adult rat, then closed with wound clips. Test substances and controls (in solution, gel, or powder form) are applied before primary closure. It is preferred to limit administration to a single application, although additional applications can be made on succeeding days by careful injection

at several sites under the incision. Wound breaking strength is evaluated between 3 and 21 days post wounding. In a second model, multiple, small, full-thickness excisions are made on the ear of a rabbit. The cartilage in the ear splints the wound, removing the variable of wound contraction from the evaluation of closure. Experimental treatments and controls are applied. The geometry and anatomy of the wound site allow for reliable quantification of cell ingrowth and epithelial migration, as well as quantitative analysis of the biochemistry of the wounds (e.g., collagen content). See, Mustoe et al., *J. Clin. Invest.* 87:694, 1991. The rabbit ear model can be modified to create an ischemic wound environment, which more closely resembles the clinical situation (Ahn et al., *Ann. Plast. Surg.* 24:17, 1990). Within a third model, healing of partial-thickness skin wounds in pigs or guinea pigs is evaluated (LeGrand et al., *Growth Factors* 8:307, 1993). Experimental treatments are applied daily on or under dressings. Seven days after wounding, granulation tissue thickness is determined. This model is preferred for dose-response studies, as it is more quantitative than other *in vivo* models of wound healing. A full thickness excision model can also be employed. Within this model, the epidermis and dermis are removed down to the panniculus carnosum in rodents or the subcutaneous fat in pigs. Experimental treatments are applied topically on or under a dressing, and can be applied daily if desired. The wound closes by a combination of contraction and cell ingrowth and proliferation. Measurable endpoints include time to wound closure, histologic score, and biochemical parameters of wound tissue. Impaired wound healing models are also known in the art (e.g., Cromack et al., *Surgery* 113:36, 1993; Pierce et al., *Proc. Natl. Acad. Sci. USA* 86:2229, 1989; Greenhalgh et al., *Amer. J. Pathol.* 136:1235, 1990). Delay or prolongation of the wound healing process can be induced pharmacologically by treatment with steroids, irradiation of the wound site, or by concomitant disease states (e.g., diabetes). Linear incisions or full-thickness excisions are most commonly used as the experimental wound. Endpoints are as disclosed above for each type of wound. Subcutaneous implants can be used to assess compounds acting in the early stages of wound healing (Broadley et al., *Lab. Invest.* 61:571, 1985; Sprugel et al., *Amer. J. Pathol.* 129: 601, 1987). Implants are prepared in a porous, relatively non-inflammatory container (e.g., polyethylene sponges or expanded polytetrafluoroethylene implants filled with bovine collagen) and placed subcutaneously in mice or rats. The interior of the implant is empty of

cells, producing a "wound space" that is well-defined and separable from the preexisting tissue. This arrangement allows the assessment of cell influx and cell type as well as the measurement of vasculogenesis/angiogenesis and extracellular matrix production.

Expression of zil1a3 proteins in animals provides models for further study of the biological effects of overproduction or inhibition of protein activity *in vivo*. Zil1a3-encoding polynucleotides can be introduced into test animals, such as mice, using viral vectors or naked DNA, or transgenic animals can be produced.

One *in vivo* approach for assaying proteins of the present invention utilizes viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, retroviruses, vaccinia virus, and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acids. For review, see Becker et al., *Meth. Cell Biol.* 43:161-89, 1994; and Douglas and Curiel, *Science & Medicine* 4:44-53, 1997. The adenovirus system offers several advantages. Adenovirus can (i) accommodate relatively large DNA inserts; (ii) be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) be used with many different promoters including ubiquitous, tissue specific, and regulatable promoters. Because adenoviruses are stable in the bloodstream, they can be administered by intravenous injection.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene is deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell (e.g., the human 293 cell line). When intravenously administered to intact animals, adenovirus primarily targets the liver. If the adenoviral delivery system has an E1 gene deletion, the virus cannot replicate in the host cells. However, the host's tissue (e.g., liver) will express and process (and, if a signal sequence is present, secrete) the heterologous protein. Secreted proteins will enter the circulation in the highly vascularized liver, and effects on the infected animal can be determined.

In another embodiment, a zil1a3 gene can be introduced in a retroviral vector as described, for example, by Anderson et al., U.S. Patent No. 5,399,346; Mann et al., *Cell* 33:153, 1983; Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., *J. Virol.* 62:1120, 1988; Temin et al., U.S. Patent No. 5,124,263; Dougherty et al., WIPO publication WO 95/07358; and Kuo et al., *Blood* 82:845, 1993. Alternatively, the vector can be introduced by "lipofection" *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7, 1987; Mackey et al., *Proc. Natl. Acad. Sci. USA* 85:8027-31, 1988). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages, including molecular targeting of liposomes to specific cells. Directing transfection to particular cell types is particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (e.g., hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

Within another embodiment target cells are removed from the the animal, and the DNA is introduced as a naked DNA plasmid. The transformed cells are then re-implanted into the body of the animal. Naked DNA vectors can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter. See, e.g., Wu et al., *J. Biol. Chem.* 267:963-7, 1992; Wu et al., *J. Biol. Chem.* 263:14621-4, 1988.

Transgenic animals, engineered to express a zil1a3 gene, and animals that exhibit a complete absence of zil1a3 gene function, referred to as "knockout mice" (Snouwaert et al., *Science* 257:1083, 1992), can be generated (Lowell et al., *Nature* 366:740-42, 1993). See also, Brinster et al., *Proc. Natl. Acad. Sci. USA* 85: 836-840, 1988; Palmiter et al., *Proc. Natl. Acad. Sci. USA* 88: 478-482, 1991; Whitelaw et al., *Transgenic Res.* 1: 3-13, 1991; and WIPO publications WO 89/01343 and WO 91/02318). Polynucleotides used in generating transgenic animals that express a zil1a3 gene will preferably contain one or more introns; genomic sequences are thus preferred.

Antisense methodology can be used to inhibit zil1a3 gene transcription to examine the effects of such inhibition *in vivo*. Polynucleotides that are complementary to a segment of a zil1a3-encoding polynucleotide (e.g., a polynucleotide as set froth in SEQ ID NO:1) are designed to bind to zil1a3-encoding mRNA and to inhibit translation of such mRNA.

For pharmaceutical use, the proteins of the present invention are formulated for local, including topical; or parenteral, including intravenous, subcutaneous, or intraperitoneal delivery according to conventional methods. Intravenous administration will be by injection or infusion. In many instances it will be beneficial to administer the protein by infusion or multiple injections per day over a period of several days to several weeks, sometimes preceded by a bolus injection. In general, pharmaceutical formulations will include a zil1a3 protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington: The Science and Practice of Pharmacy, Gennaro, ed., Mack Publishing Co., Easton, PA, 19th ed., 1995.

As noted above, inhibition of IL-1 activity requires a large molar excess of antagonist. Doses of zil1a3 antagonist proteins will in general be quite large, particularly when treating life-threatening conditions. IL-1ra appears safe in high doses. Thus, doses of zil1a3 antagonist proteins will range from as low as 10 mg per patient per day to as high as 100 mg or more per hour infused over a period of days. Doses of IL-1ra found to be efficacious in clinical studies include 70 mg per patient per day in rheumatoid arthritis and up to 3,400 mg per patient per day in graft-versus-host disease. The exact dose will be determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. The proteins may be administered for acute treatment, over one week or less, but will often be used in treatment of chronic conditions requiring administration over several weeks to several months or longer. In general, a therapeutically effective amount of a zil1a3 protein is an amount sufficient to produce a clinically significant improvement in one or more

standard indicators appropriate to the treated condition. Therapeutic endpoints will be apparent to those skilled in the art.

Zil1a3 proteins, both agonist and antagonist, can be used as standards in assays of IL-1 and IL-1 inhibitor. Such assays can comprise any of a number of standard formats, include radioreceptor assays and ELISAs. Zil1a3 protein standards can be prepared in labeled form using a radioisotope, enzyme, fluorophore, or other compound that produces a detectable signal. The proteins can be packaged in kit form, such kits comprising one or more vials containing the zil1a3 protein and, optionally, a diluent, an antibody, a labeled binding protein, etc.

Assay kits can also be used in the research laboratory to detect IL-1 and IL-1 inhibitor activities produced by cultured cells or test animals.

Zil1a3 proteins are also useful as research reagents. For example, zil1a3 agonist proteins can be used as cell culture components to promote the growth of IL-1 responsive cells, including fibroblasts, smooth muscle cells, and mesangial cells. Agonists can be combined with IL-3 and other cytokines to expand $CD34^+$ peripheral blood cells, and with IL-3 and IL-6 to promote the proliferation of stem cells.

The invention further provides polypeptides that comprise an epitope-bearing portion of a protein as shown in SEQ ID NO:7. An "epitope" is a region of a protein to which an antibody can bind. See, for example, Geysen et al., *Proc. Natl. Acad. Sci. USA* 81:3998-4002, 1984. Epitopes can be linear or conformational, the latter being composed of discontinuous regions of the protein that form an epitope upon folding of the protein. Linear epitopes are generally at least 6 amino acid residues in length. Relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, Sutcliffe et al., *Science* 219:660-666, 1983. Antibodies that recognize short, linear epitopes are particularly useful in analytic and diagnostic applications that employ denatured protein, such as Western blotting (Tobin, *Proc. Natl. Acad. Sci. USA* 76:4350-4356, 1979).

Antigenic, epitope-bearing zil1a3 polypeptides useful for raising antibodies, including monoclonal antibodies, that specifically bind to a zil1a3 protein. Antigenic, epitope-bearing polypeptides contain a sequence of at least six, preferably at least nine, more preferably from 15 to about 30 contiguous amino acid residues of a zil1a3 protein (e.g., SEQ ID NO:2 or SEQ

ID NO:8). Polypeptides comprising a larger portion of a zil1a3 protein, i.e. from 30 to 50 residues up to the entire sequence are included. It is preferred that the amino acid sequence of the epitope-bearing polypeptide is selected to provide substantial solubility in aqueous solvents, that is the sequence includes relatively hydrophilic residues, and hydrophobic residues are substantially avoided. Sequences containing proline residues are preferred. Preferred such regions include residues 10-15, 38-43, 91-96, 92-97, and 124-129 of SEQ ID NO:2. As noted above, it is generally preferred to use somewhat longer peptides as immunogens, such as a peptide comprising residues 91-104 SEQ ID NO:2.

As used herein, the term "antibodies" includes polyclonal antibodies, monoclonal antibodies, antigen-binding fragments thereof such as $F(ab')_2$ and Fab fragments, single chain antibodies, and the like, including genetically engineered antibodies. Non-human antibodies can be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. One skilled in the art can generate humanized antibodies with specific and different constant domains (i.e., different Ig subclasses) to facilitate or inhibit various immune functions associated with particular antibody constant domains. Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to a zil1a3 protein, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled zil1a3 polypeptide). Antibodies are defined to be specifically binding if they bind to a zil1a3 protein with an affinity at least 10-fold greater than the binding affinity to control (non-zil1a3) polypeptide. It is preferred that the antibodies exhibit a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The affinity of a monoclonal antibody can be readily determined by one of ordinary skill in the art (see, for example, Scatchard, *Ann. NY Acad. Sci.* 51: 660-672, 1949).

Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see for example, Hurrell, J. G. R., Ed., *Monoclonal Hybridoma Antibodies: Techniques and Applications*, CRC Press, Inc., Boca Raton, FL, 1982). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats. The immunogenicity of a zil1a3 protein may be increased through the use of an adjuvant such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of a zil1a3 protein or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

A variety of assays known to those skilled in the art can be used to detect antibodies that specifically bind to a zil1a3 protein. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual*, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include concurrent immunoelectrophoresis, radio-immunoassays, radio-immunoprecipitations, enzyme-linked immunosorbent assays (ELISA), dot blot assays, Western blot assays, inhibition or competition assays, and sandwich assays.

Antibodies to zil1a3 may be used for affinity purification of zil1a3 proteins; within diagnostic assays for determining circulating levels of zil1a3 proteins; for detecting or quantitating soluble zil1a3 protein as a marker of underlying pathology or disease; for immunolocalization within whole animals or tissue sections, including immunodiagnostic applications; for immunohistochemistry; for screening expression libraries; and for other uses that will be evident to those skilled in the art. For certain applications, including *in vitro* and *in vivo* diagnostic uses, it is advantageous to employ labeled antibodies. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or

labels may feature use of biotin-avidin or other complement/anti-complement pairs as intermediates.

Zil1a3 polynucleotides can be used to determine the presence of mutations at or near the zil1a3 locus on human chromosome 2 (2q14). Detectable chromosomal aberrations at the zil1a3 gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes, and rearrangements. These aberrations can occur within the coding sequence, within introns, or within flanking sequences, including upstream promoter and regulatory regions, and may be manifested as physical alterations within a coding sequence or changes in gene expression level. Analytical probes will generally be at least 20 nucleotides in length, although somewhat shorter probes (14-17 nucleotides) can be used. PCR primers are at least 5 nucleotides in length, preferably 15 or more nt, more preferably 20-30 nt. Short polynucleotides can be used when a small region of the gene is targetted for analysis. For gross analysis of genes, a polynucleotide probe may comprise an entire exon or more. Probes will generally comprise a polynucleotide linked to a signal-generating moiety such as a radionucleotide. In general, these diagnostic methods comprise the steps of (a) obtaining a genetic sample from a patient; (b) incubating the genetic sample with a polynucleotide probe or primer as disclosed above, under conditions wherein the polynucleotide will hybridize to complementary polynucleotide sequence, to produce a first reaction product; and (c) comparing the first reaction product to a control reaction product. A difference between the first reaction product and the control reaction product is indicative of a genetic abnormality in the patient. Genetic samples for use within the present invention include genomic DNA, cDNA, and RNA. The polynucleotide probe or primer can be RNA or DNA, and will comprise at least a portion of SEQ ID NO:1, the complement of SEQ ID NO:1, or an RNA equivalent thereof. Suitable assay methods in this regard include molecular genetic techniques known to those in the art, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, ligation chain reaction (Barany, *PCR Methods and Applications* 1:5-16, 1991), ribonuclease protection assays, and other genetic linkage analysis techniques known in the art (Sambrook et al., *ibid.*; Ausubel et. al., *ibid.*; A.J. Marian, *Chest* 108:255-65, 1995). Ribonuclease protection assays (see, e.g., Ausubel et al., *ibid.*, ch. 4) comprise the

hybridization of an RNA probe to a patient RNA sample, after which the reaction product (RNA-RNA hybrid) is exposed to RNase. Hybridized regions of the RNA are protected from digestion. Within PCR assays, a patient genetic sample is incubated with a pair of polynucleotide primers, and the region between the primers is amplified and recovered. Changes in size or amount of recovered product are indicative of mutations in the patient. Another PCR-based technique that can be employed is single strand conformational polymorphism (SSCP) analysis (Hayashi, *PCR Methods and Applications* 1:34-38, 1991).

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1

Recombinant human zil1a3 was produced in *E. coli* using a His_6 tag/maltose binding protein (MBP) double affinity fusion system as generally disclosed by Pryor and Leiting, *Prot. Expr. Pur.* 10:309-319, 1997. A thrombin cleavage site was placed at the junction between the affinity tag and zil1a3 sequences.

The fusion construct was assembled in the vector pTAP98, which comprised sequences for replication and selection in *E. coli* and yeast, the *E. coli* tac promoter, and a unique SmaI site just downstream of the MBP-His_6-thrombin site coding sequences. The zil1a3 cDNA (SEQ ID NO:1) was amplified by PCR using primers each comprising 40 bp of sequence homologous to vector sequence and 25 bp of sequence that annealed to the cDNA. The reaction was run using Pwo DNA polymerase (Boehringer Mannheim, Indianapolis, IN) for 30 cycles of 94°C, 30 seconds; 60°C, 60 seconds; and 72°C, 60 seconds. One microgram of the resulting fragment was mixed with 100 ng of SmaI-cut pTAP98, and the mixture was transformed into yeast to assemble the vector by homologous recombination (Oldenburg et al., *Nucl. Acids. Res.* 25:451-452, 1997). Ura^+ transformants were selected.

Plasmid DNA was prepared from yeast transformants and transformed into *E. coli* MC1061. Pooled plasmid DNA was then prepared from the MC1061 transformants by the miniprep method after scraping an

entire plate. Plasmid DNA was analyzed by restriction digestion using NcoI and EcoRI.

E. coli strain BL21 was used for expression of zil1a3. Cells were transformed by electroporation and grown on minimal glucose plates containing casamino acids and ampicillin.

Protein expression was analyzed by gel electrophoresis. Cells were grown in liquid media containing ampicillin. After one hour at 37°C, IPTG was added to a final concentration of 1 mM, and the cells were grown for an additional 2-3 hours at 37°C. Cells were disrupted using glass beads, and extracts were prepared. Seven of nine isolates were found to produce large amounts of the fusion protein.

Example 2

Expression levels of zil1a3 were compared in *E. coli* strains BL21, DH10B™ (Life Technologies, Inc., Gaithersburg, MD), MC1061, and TOP10. Cells were grown in duplicate cultures in minimal glucose media containing casamino acids and 100 μg/ml ampicillin. One culture from each set was induced by the addition of 100 mM IPTG for 2-3 hours. Extracts were prepared and analyzed by gel electrophoresis as in Example 1. The order of expression level was BL21 > MC1061 >> DH10B™ > TOP10.

Example 3

Larger scale cultures of zil1a3 *E. coli* BL21 transformants were prepared by the method of Pryor and Leiting (*ibid.*). 100-ml cultures in minimal glucose media containing casamino acids and 100 μg/ml ampicillin were grown at 37°C in 500-ml baffled flasks to $OD_{600} \approx 0.5$. Cells were harvested by centrifugation and resuspended in 100 ml of the same media at room temperature. After 15 minutes, IPTG was added to 0.5 mM, and cultures were incubated at room temperature (ca. 22.5°C) for 16 to 20 hours with shaking at 125 rpm. Two ml of each culture was set aside for subcellular fractionation, and the remaining culture was harvested by centrifugation. Cell pellets were stored at -70°C. Analysis of cell lysates showed that most zil1a3 protein was in the soluble fraction.

Example 4

For larger-scale protein preparation, 500-ml cultures of *E. coli* BL21 expressing the zil1a3-MBP-His_6 fusion protein are prepared essentially as disclosed in Example 3. Cell pellets are resuspended in 100 ml of binding buffer (20 mM Tris, pH 7.58, 100 mM NaCl, 20 mM NaH_2PO_4, 0.4 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride [Pefabloc® SC; Boehringer-Mannheim], 2 μg/ml Leupeptin, 2 μg/ml Aprotinin). The cells are lysed in a French press at 30,000 psi, and the lysate is centrifuged at 18,000 x g for 45 minutes at 4°C to clarify it. Protein concentration is estimated by gel electrophoresis with a BSA standard.

Recombinant zil1a3 fusion protein is purified from the lysate by affinity chromatography. Immobilized cobalt resin (Talon® resin; Clontech Laboratories, Inc., Palo Alto, CA) is equilibrated in binding buffer. One ml of packed resin per 50 mg protein is combined with the clarified supernatant in a tube, and the tube is capped and sealed, then placed on a rocker overnight at 4°C. The resin is then pelleted by centrifugation at 4°C and washed three times with binding buffer. Protein is eluted with binding buffer containing 0.2 M imidazole. The resin and elution buffer are mixed for at least one hour at 4°C, the resin is pelleted, and the supernatant is removed. An aliquot is analyzed by gel electrophoresis, and concentration is estimated. Amylose resin is equilibrated in amylose binding buffer (20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM EDTA) and combined with the supernatant from the Talon resin at a ratio of 2 mg fusion protein per ml of resin. Binding and washing steps are carried out as disclosed above. Protein is eluted with amylose binding buffer containing 10 mM maltose using as small a volume as possible to minimize the need for subsequent concentration. The eluted protein is analyzed by gel electrophoresis and staining with Coomassie blue using a BSA standard, and by Western blotting using an anti-MBP antibody.

Example 5

Zil1a3 was mapped to human chromosome 2 using the commercially available version of the Stanford G3 Radiation Hybrid Mapping Panel (Research Genetics, Inc., Huntsville, AL). This panel contains PCRable DNAs from each of 83 radiation hybrid clones of the whole human genome, plus two control DNAs (the RM donor and the A3 recipient). A publicly

available WWW server (http://shgc-www.stanford.edu) was used for chromosomal localization of markers.

For the mapping of the zil1a3 gene, 20-µl reaction mixtures were set up in a PCRable 96-well microtiter plate (Stratagene, La Jolla, CA) and used in a thermal cycler (RoboCycler® Gradient 96; Stratagene, La Jolla, CA). Each of the 85 PCR reaction mixtures consisted of 2 µl buffer (10X KlenTaq PCR reaction buffer, Clontech Laboratories, Inc., Palo Alto, CA), 1.6 µl dNTPs mix (2.5 mM each, PERKIN-ELMER, Foster City, CA), 1 µl sense primer, ZC 20,354 (SEQ ID NO:11), 1 µl antisense primer, ZC 20,355 (SEQ ID NO:12), 2 µl of a density increasing agent and tracking dye (RediLoad™, Research Genetics, Inc., Huntsville, AL), 0.4 µl of a commercially available DNA polymerase/antibody mix (50X Advantage™ KlenTaq Polymerase Mix, obtained from Clontech Laboratories, Inc.), 25 ng of DNA from an individual hybrid clone or control and x µl ddH_2O for a total volume of 20 µl. The mixtures were overlaid with an equal amount of mineral oil and sealed. The PCR cycler conditions were as follows: an initial 5-minute denaturation at 94°C; 35 cycles of 45 seconds denaturation at 94°C, 45 seconds annealing at 64°C, and 75 seconds extension at 72°C; followed by a final extension of 7 minutes at 72°C. The reactions were separated by electrophoresis on a 2% agarose gel.

The results showed linkage of the zil1a3 gene to the human chromosome 2 framework marker AFMa037xf1 with a LOD score of 13 and at a distance of 4.65 cR_10000 from the marker. The use of surrounding genes/markers placed Zil1a3 in the 2q14 chromosomal region. The 2q14 region also contains the genes for interleukin 1 alpha (IL-1α), interleukin 1 beta (IL-1β), and the interleukin 1 receptor antagonist (IL-1ra). The zil1a3 gene maps in close proximity to IL-1ra (~215.7 kb proximal of IL-1ra).

Example 6

Northern blot analysis was performed using human multiple tissue blots (MTN® I, MTN® II, and MTN® III) (Clontech Laboratories, Inc., Palo Alto, CA) and human fetal multiple tissue blots (Clontech Laboratories, Inc.). A 595-bp human probe was generated by amplification of an XhoI-NcoI cDNA fragement. The 595-bp fragment was gel purified using a spin column containing a silica gel membrane (QIAquick™ Gel Extraction Kit; Qiagen, Inc., Valencia, CA). The probe was radioactively labeled with ^{32}P using a

commercially available kit (Rediprime™ II random-prime labeling system; Amersham Corp., Arlington Heights, IL) according to the manufacturer's specifications. The probe was purified using a push column (NucTrap® column; Stratagene, La Jolla, CA). A commercially available hybridization solution (ExpressHyb™ Hybridization Solution; Clontech Laboratories, Inc.) was used for the hybridizing solution for the blots. Hybridization took place overnight at 65°C. The blots were then washed 4 times in 2X SCC and 0.05% SDS at room temperature, followed by two washes in 0.1X SSC and 0.1% SDS at 50°C. Two transcripts were detected at approximately 1.8kb and 2.8kb in placenta. Signal intensity was strong for placenta. A faint signal was seen at 2.8 kb in testis, thyroid, spinal cord, and trachea.

Dot blots (Human RNA Master Blots™; Clontech Laboratories, Inc.) were analyzed essentially as disclosed above. A signal was seen in placenta.

Example 7

Human northern blots (MTN® I, MTN® II, MTN® III, and human fetal multiple tissue blots; Clontech Laboratories, Inc.) were analyzed with a mouse zil1a3 probe. A 350-bp mouse probe was generated from a mouse cDNA (SEQ ID NO:14) by PCR using oligonucleotide primers zc18,609 (SEQ ID NO:15) and zc18,610 (SEQ ID NO:16). The reaction was run at 94°C for 2 minutes; then 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 5 minutes. The PCR product was gel purified using a spin column containing a silica gel membrane. The probe was radioactively labeled with ^{32}P using a commercially available kit (Rediprime™ II random-prime labeling system; Amersham Corp.) according to the manufacturer's specifications. The probe was purified using a push column. Hybridization and washing were carried out essentially as disclosed in Example 6, except the final wash was perfomed four times. Two transcripts, of approximately 1.0 kb and 1.5 kb, were seen in heart, brain, lung, liver, skeletal muscle, kidney, pancreas, adrenal gland, spinal cord, peripheral blood leukocytes, colon, small intestine, testis, and prostate. Signal intensity was strongest in heart and liver.

Dot blots (Human RNA Master Blots™; Clontech Laboratories, Inc.) were also analyzed by essentially the same procedures. Signals were seen in placenta, kidney, liver, and adrenal gland.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. An isolated protein comprising a sequence of amino acid residues as shown in SEQ ID NO:7.

2. The isolated protein of claim 1 wherein the protein comprises a Lys residue at position 148 of SEQ ID NO:7.

3. The isolated protein of claim 1 wherein the protein comprises an Asp residue at position 148 of SEQ ID NO:7.

4. The isolated protein of claim 1 wherein the protein comprises the amino acid sequence of SEQ ID NO:8.

5. The isolated protein of any of claims 1 - 4 which is 155 amino acid residues in length.

6. The isolated protein of any of claims 1 - 4 wherein the protein is from 155 to 1200 amino acid residues in length.

7. An isolated polypeptide of at least 15 amino acid residues comprising an epitope-bearing portion of a protein of SEQ ID NO:7.

8. An expression vector comprising the following operably linked elements:

(a) a transcription promoter;

(b) a DNA segment encoding a protein according to any of claims 1 - 6; and

(c) a transcription terminator.

9. The expression vector of claim 8 further comprising a secretory signal sequence operably linked to the DNA segment.

10. A cultured cell comprising an expression vector according to claim 8 or claim 9.

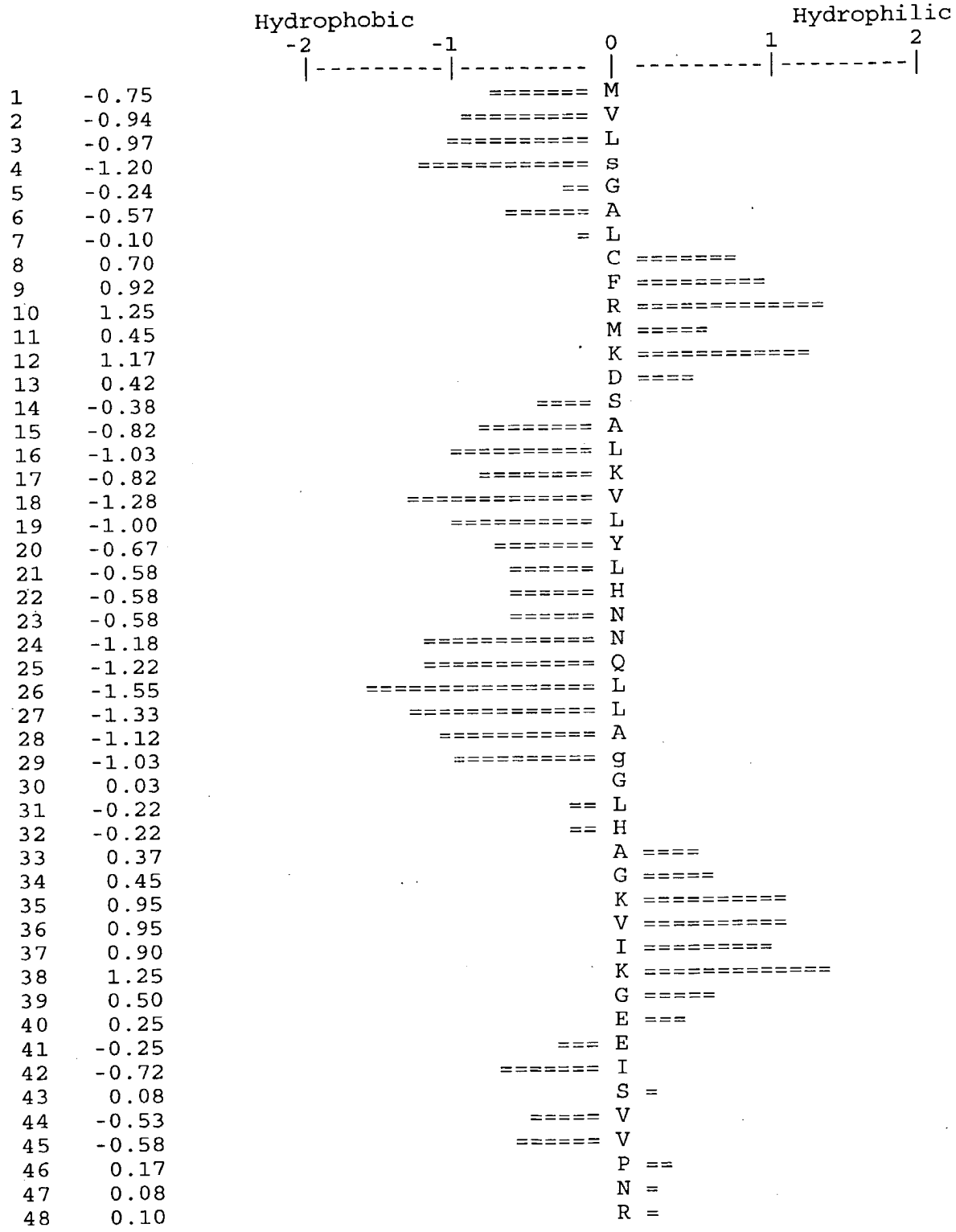
11. A method of making a protein comprising:

culturing a cell according to claim 10 under conditions wherein the DNA segment is expressed; and

recovering the protein encoded by the DNA segment.

12. An antibody that specifically binds to the protein of claim 1.

13. A method of modulating an immune response in an animal comprising administering to the animal a composition comprising the protein of any of claims 1 - 6 in combination with a pharmaceutically acceptable vehicle.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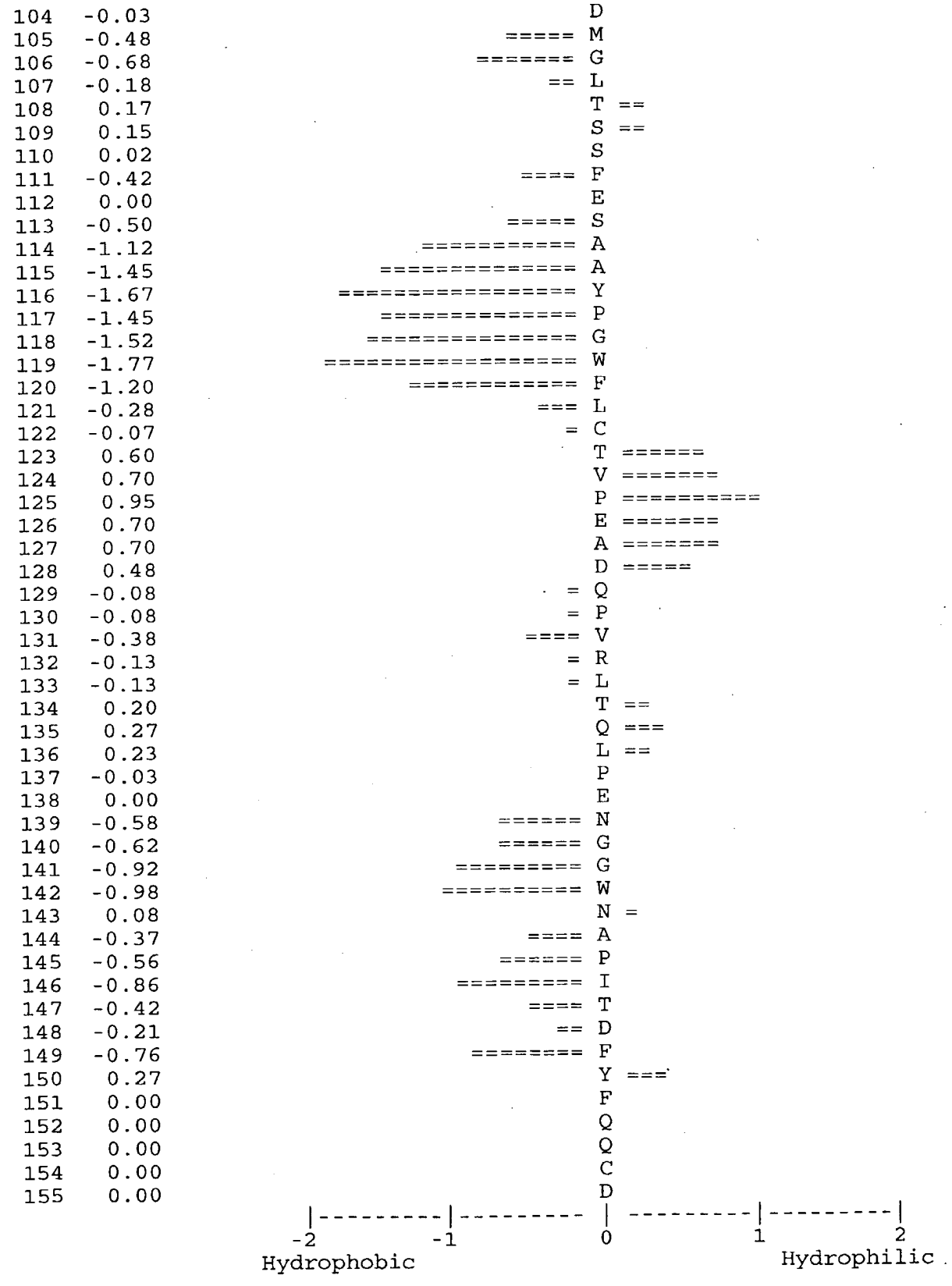
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Ser Phe Thr Phe Tyr Arg Arg Asp Met Gly Leu Thr Ser Ser Phe Glu
100 105 110
Ser Ala Ala Tyr Pro Gly Trp Phe Leu Cys Thr Val Pro Glu Ala Asp
115 120 125

Gln Pro Val Arg Leu Thr Gln Leu Pro Glu Asn Gly Gly Trp Asn Ala
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Pro Ile Thr Lys Phe Tyr Phe Gln Gln Cys Asp
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<223> n = A,T,C or G

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garathwsng tngtnccnaa ymgntggytn gaygcnwsny tnwsnccngt nathytnggn 180
gtncarggng gnwsncartg yytnwsntgy ggngtnggnc argarccnac nytnacnytn 240
garccngtna ayathatgga rytntayytn ggngcnaarg arwsnaarws nttyacntty 300
taymgnmgng ayatgggnyt nacnwsnwsn ttygarwsng cngcntaycc nggntggtty 360
ytntgyacng tnccngargc ngaycarccn gtnmgnytna cncarytncc ngaraayggn 420
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<220>
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<223> n = A,T,C or G

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garathwsng tngtnccnaa ymgntggytn gaygcnwsny tnwsnccngt nathytnggn 180
gtncarggng gnwsncartg yytnwsntgy ggngtnggnc argarccnac nytnacnytn 240
garccngtna ayathatgga rytntayytn ggngcnaarg arwsnaarws nttyacntty 300

taymgnmgng ayatgggnyt nacnwsnwsn ttygarwsng cngcntaycc nggntggtty 360
ytntgyacng tnccngargc ngaycarccn gtnmgnytna cncarytncc ngaraayggn 420
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<220>
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<222> (101)...(571)

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                                              Met Met Val Leu Ser
                                               1               5

ggg gca cta tgc ttc cga atg aag gat tca gcc ttg aag gta ctg tat       163
Gly Ala Leu Cys Phe Arg Met Lys Asp Ser Ala Leu Lys Val Leu Tyr
                10                  15                  20

ctg cac aat aac cag ctg ctg gct gga gga ctg cac gca gag aag gtc       211
Leu His Asn Asn Gln Leu Leu Ala Gly Gly Leu His Ala Glu Lys Val
            25                  30                  35

att aaa ggt gag gag atc agt gtt gtc cca aat cgg gca ctg gat gcc       259
Ile Lys Gly Glu Glu Ile Ser Val Val Pro Asn Arg Ala Leu Asp Ala
        40                  45                  50

agt ctg tcc cct gtc atc ctg ggc gtt caa gga gga agc cag tgc cta       307
Ser Leu Ser Pro Val Ile Leu Gly Val Gln Gly Gly Ser Gln Cys Leu
    55                  60                  65

tct tgt ggg aca gag aaa ggg cca att ctg aaa ctt gag cca gtg aac       355
Ser Cys Gly Thr Glu Lys Gly Pro Ile Leu Lys Leu Glu Pro Val Asn
70                  75                  80                  85

atc atg gag ctc tac ctc ggg gcc aag gaa tca aag agc ttc acc ttc       403
Ile Met Glu Leu Tyr Leu Gly Ala Lys Glu Ser Lys Ser Phe Thr Phe
                90                  95                  100

tac cgg cgg gat atg ggt ctt acc tcc agc ttc gaa tcc gct gcc tac       451
Tyr Arg Arg Asp Met Gly Leu Thr Ser Ser Phe Glu Ser Ala Ala Tyr
            105                 110                 115

cca ggc tgg ttc ctc tgc acc tca ccg gaa gct gac cag cct gtc agg       499
Pro Gly Trp Phe Leu Cys Thr Ser Pro Glu Ala Asp Gln Pro Val Arg
        120                 125                 130

ctc act cag atc cct gag gac ccc gcc tgg gat gct ccc atc aca gac       547
Leu Thr Gln Ile Pro Glu Asp Pro Ala Trp Asp Ala Pro Ile Thr Asp
    135                 140                 145
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<400> 16
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INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 99/23533

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/25 C07K14/545 C07K16/24 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 541 920 A (SYNERGEN INC) 19 May 1993 (1993-05-19)	
P,X	J.J. MULERO ET AL: "IL1HY1: a novel Interleukin-1 receptor antagonist gene" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS., vol. 263, no. 3, 5 October 1999 (1999-10-05), pages 702-706, XP002126222 ACADEMIC PRESS INC. ORLANDO, FL., US ISSN: 0006-291X the whole document	1,3-13
P,X	WO 99 35268 A (IMMUNEX CORP ;SIMS JOHN ERNEST (US)) 15 July 1999 (1999-07-15) claims; examples 1-3 Sequences ID no. 3 and 4	1,3-13

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 2000	08/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Le Cornec, N

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 99/23533

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 98 47921 A (SCHERING CORP) 29 October 1998 (1998-10-29) Sequences ID no. 1 and 2 claims; examples	1,3-13
E	WO 99 51744 A (HYSEQ INC) 14 October 1999 (1999-10-14) sequence ID no. 5 page 6 page 12, line 23 -page 14 examples 6,10,12	1,3-13
T	SMITH D.E. ET AL: "Four new members expand the iL-1 superfamily" EMBL DATBASE ENTRY AAF25210, ACCESSION NUMBER AAF25210, 27 January 2000 (2000-01-27), XP002131487 abstract & JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 275, no. 2, January 2000 (2000-01), pages 1169-1175, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD., US ISSN: 0021-9258	1,3-5, 7-11

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/23533

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 13 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern nal Application No
PCT/US 99/23533

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0541920 A	19-05-1993	AT 88502 T	15-05-1993
		AU 633831 B	11-02-1993
		AU 3746989 A	12-12-1989
		DD 283835 A	24-10-1990
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		ES 2018750 T	16-10-1994
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		IE 66712 B	24-01-1996
		JP 3505279 T	21-11-1991
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		MX 16206 A	01-11-1993
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		NZ 229326 A	23-12-1992
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		US 5075222 A	24-12-1991
		ZA 8904052 A	25-04-1990
		PL 164003 B	30-06-1994
WO 9935268 A	15-07-1999	AU 2110499 A	26-07-1999
WO 9847921 A	29-10-1998	AU 7103198 A	13-11-1998
		EP 0977779 A	09-02-2000
WO 9951744 A	14-10-1999	AU 3449599 A	25-10-1999